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Forskolin stimulates pinealocyte cGMP accumulation

Dramatic potentiation by an α_1-adrenergic → $[Ca^{2+}]_i$ mechanism involving protein kinase C

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The effect of forskolin on cGMP regulation was investigated using dispersed rat pinealocytes. Forskolin stimulated cGMP accumulation in a concentration-dependent manner; this response was strongly potentiated by an α_1-adrenergic → $[Ca^{2+}]_i$ mechanism involving protein kinase C. These findings provide further evidence that activation of two receptor-regulated signal transduction mechanisms may be commonly required for maximal stimulation of cGMP accumulation, and establish a new experimental approach to the study of cGMP regulation.

Forskolin; cyclic GMP; Guanylyl cyclase; Protein kinase C; Ca^{2+}; (Pineal)

1. INTRODUCTION

Activation of biochemical 'AND' gates [The term biochemical AND gate describes regulatory mechanisms which require that two independent receptor-regulated processes must act simultaneously to produce a full response. This is analogous to the electronic AND gate which requires that two electronic signals must be received simultaneously for an output signal to be generated.] generates large and highly selective changes in cytosolic concentrations of cyclic nucleotides [1–5]. Examples come from the rat pinealocyte where norepinephrine (NE) stimulates cAMP and cGMP accumulation by concurrent activation of α_1- and β-adrenoceptors [1]. β-Adrenoceptor activation produces an increase of 7–10-fold in cAMP and of 2–4- fold in cGMP. Selective α_1-adrenoceptor stimulation alone has no effect on cAMP or cGMP, but potentiates β-adrenergic stimulation of cAMP by about 10-fold and β-adrenergic stimulation of cGMP about 50–100-fold [6]. These interactions generate > 100-fold increases in pinealocyte cyclic nucleotides.

The post-receptor mechanism involved in the α_1-adrenergic potentiation of β-adrenergic stimulation of cAMP and cGMP appears to involve α_1-adrenergic activation of both Ca^{2+}/phospholipid-dependent protein kinase C (PKC) [7,8] and elevation of intracellular Ca^{2+} ($[CA^{2+}]_i$) [9,10]. The available evidence suggests that interaction between PKC and Ca^{2+} may act in part to enhance activation of cyclases by direct phosphorylation [11–14]. In the case of cGMP, the potentiation mechanism also requires a distinct second Ca^{2+}-dependent event [10].

Here, we have extended our investigation of the control of pinealocyte cGMP using forskolin (FSK)

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[15,16], which has the well documented action of directly activating adenylyl cyclase and elevating cAMP. As a result, a conceptual FSK = cAMP equation has evolved, reflecting the widespread belief that FSK modifies cellular physiology through cAMP [17]. However, reports of small effects of FSK on cGMP have also appeared [18,19]. We confirmed this with the rat pinealocyte and also found that the cGMP response to FSK is strongly potentiated by an α_1-adrenergic → $[Ca^{2+}]_i$ mechanism involving PKC.

2. EXPERIMENTAL

2.1. *Materials*

Phenylephrine (PE), propranolol, nifedipine, diolein, dioctanoylglycerol, and A23187 were obtained from Sigma (St. Louis, MO). Phorbol esters and FSK were purchased from Calbiochem (La Jolla, CA). All other drugs and chemicals were from commercial sources and of the purest grade available. Antibodies for the cAMP and cGMP radioimmunoassays were gifts from Dr K. Catt (NICHD, NIH, Bethesda, MD). Sprague-Dawley rats (female, 200 g) were obtained from Charles River.

2.2. *Methods*

Pinealocytes were prepared and treated as described [6,20]. Drugs were dissolved in water, ethanol or dimethyl sulfoxide and added at less than 1% of the total volume. At this concentration, ethanol or dimethyl sulfoxide has no effect on the cAMP and cGMP responses of pinealocytes to NE. After treatment, cells were pelleted and frozen [6].

The frozen cell pellet was lysed by boiling for 3 min in 5 mM acetic acid (100 μl). The preparation was then centrifuged (12000 × g, 10 min), and the supernatant used for determinations of cAMP and cGMP by radioimmunoassay [6,21] and protein [22].

Triplicate samples were used for each group; each sample was analyzed in duplicate and the average of duplicate determinations was used to generate the mean ± SE of cyclic nucleotides in each treatment group. Data were analyzed by Bartlett's test for heterogeneity and Duncan's multiple range test [23].

3. RESULTS

3.1. *Effect of FSK on cAMP and cGMP accumulation*

FSK treatment increased both cAMP and cGMP in a time- and concentration-dependent manner (fig.1); the largest response to FSK alone was about 10-fold, which represents about 10% of the maximal responses normally seen with NE [6]. Responses of this magnitude are similar to the partial responses elicited by agents which are thought to stimulate adenylyl and guanylyl cyclase activities via an action on G_s, the GTP-binding regulatory protein; these agents include the β-adrenergic agonist isoproterenol (ISO), vasoactive intestinal peptide (VIP), and cholera toxin (CT) [6,10, 24–28].

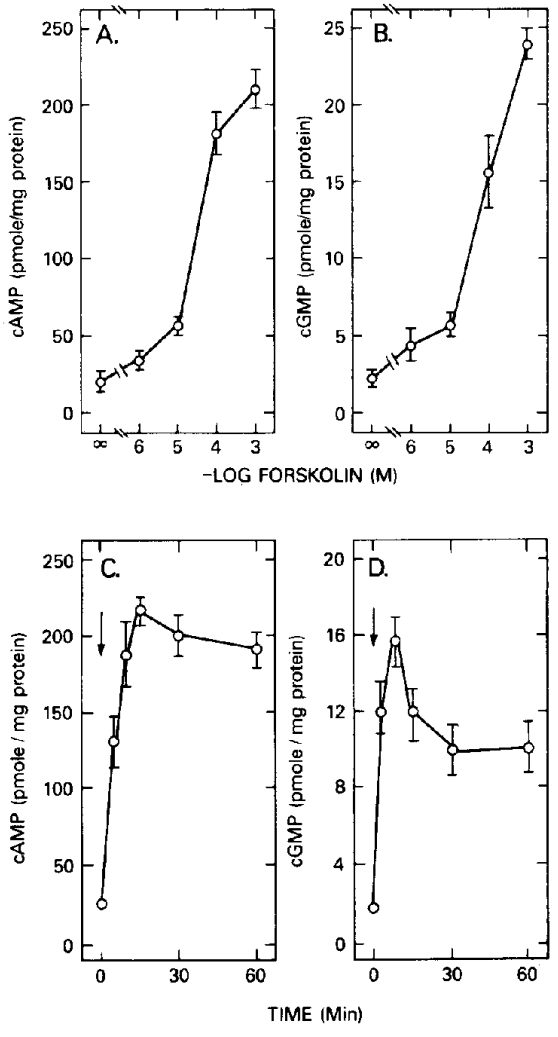

Fig.1. FSK stimulation of cAMP and cGMP accumulation in rat pinealocytes. The absence of an error bar indicates that SE fell within the symbol. (A,B) Concentration-response studies in which cells were incubated for 15 min with the indicated concentration of FSK. (C,D) Time-course studies in which pinealocytes were incubated for 0–30 min with FSK (100 μM). The arrow indicates when FSK was added.

3.2. *Effect of phenylephrine(PE) on cAMP and cGMP accumulation in FSK treated pinealocytes*

Maximal cAMP and cGMP responses can be

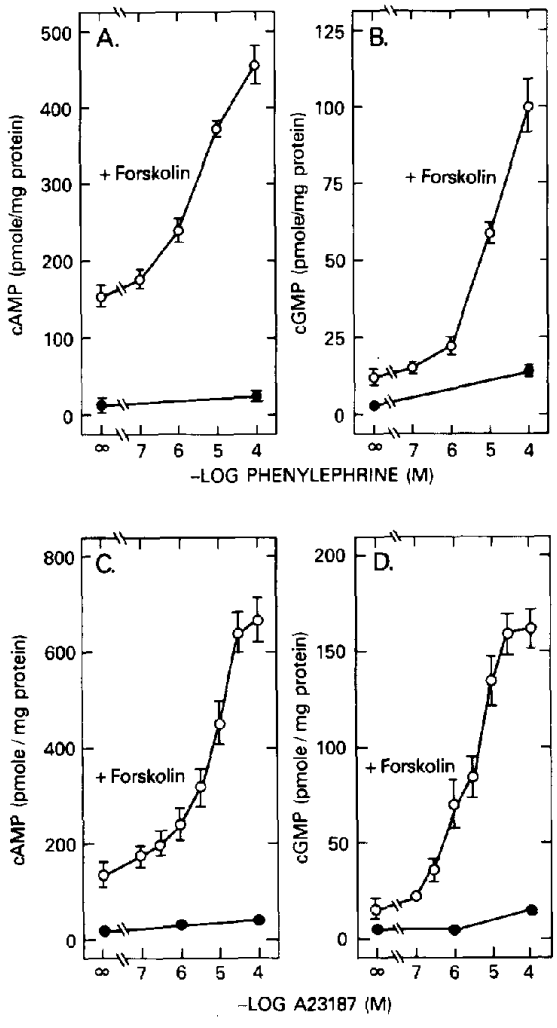

Fig.2. Effect of phenylephrine or A23187 on cAMP and cGMP accumulation in FSK (100 μM)-treated pinealocytes. Pinealocytes were incubated as indicated for 15 min. (A,B) Cells were treated with FSK and the indicated concentrations of phenylephrine; propranolol (10 μM) was present to block possible β-adrenergic effects. (C,D) Cells were treated with FSK and the indicated concentrations of A23187.

produced by treatment with the mixed α_1,β-adrenergic agonist NE or by treatment with either ISO, VIP or CT in combination with α_1-adrenergic agonists, such as PE [6,10,24–28]. PE also potentiates stimulation of cAMP by FSK [14]. We were interested in determining whether α_1-adrenergic activation could potentiate FSK stimulation of cGMP accumulation. Alone, PE had only a small effect on cGMP (fig.2A,B). However, it potentiated FSK stimulation of cGMP in a concentration-dependent manner. This observation is consistent with the interpretation that FSK shares a common mechanism of action in cGMP regulation with ISO, VIP and CT [29].

3.3. *Effect of A23187 or K^+ on cAMP and cGMP accumulation in FSK treated pinealocytes*

PE is thought to potentiate β-adrenergic, VIP and CT stimulation of pineal cyclic nucleotides by increasing Ca^{2+} influx, thereby elevating $[Ca^{2+}]_i$ about 4-fold [9,30]. To determine whether the effects of FSK were potentiated by elevating $[Ca^{2+}]_i$, we treated cells with FSK and either A23187 or K^+. The former is a Ca^{2+} ionophore. The latter elevates $[Ca^{2+}]_i$ indirectly by depolarizing the cell; this opens voltage-sensitive Ca^{2+} channels. In these studies A23187 (fig.2C,D) or K^+ (fig.3A,B) potentiated FSK stimulation of both cAMP and cGMP accumulation.

The issue of whether K^+ was acting on the cGMP response through Ca^{2+} was further investigated by determining if nifedipine (NIF) could block this effect. NIF blocks Ca^{2+} influx through some voltage-sensitive Ca^{2+} channels [31]; in the pinealocyte it inhibits K^+-induced increases in $[Ca^{2+}]_i$ and K^+-induced potentiation of cAMP and cGMP responses to ISO, VIP or CT [9,30]. In the present experiments (table 1, expt I) NIF blocked the K^+-induced potentiation of FSK stimulation of cAMP and cGMP accumulation. This indicates that K^+ was probably acting through Ca^+. These results are consistent with the interpretation that the cGMP response to FSK is potentiated by PE acting via a Ca^{2+} mechanism.

3.4. *Effect of K^+ or 4β-phorbol 12-myristate 13-acetate (PMA) on cAMP and cGMP accumulation in FSK treated pinealocytes*

Ca^{2+} is known to potentiate stimulation of pineal cyclic nucleotides in part by translocation of PKC [7,8,32]; in the case of cGMP potentiation, Ca^{2+} activates another process [10]. We studied the role of PKC using cells treated with concentrations of FSK and K^+ (15 mM) which alone produced only a small elevation of cAMP and cGMP (fig.3C,D). In the presence of FSK alone, the PKC activator PMA potentiated FSK stimulation of cAMP as reported in [14]; in contrast, cGMP was not influenced. However, in the presence of K^+ (15

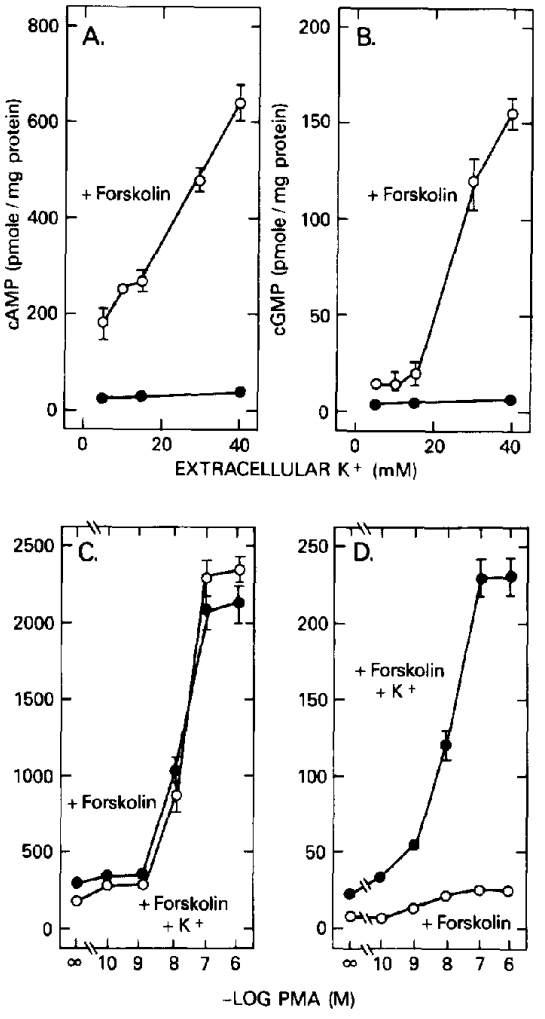

Fig.3. Effect of K^+ or PMA on cAMP and cGMP accumulation in FSK (100 μM)-treated pinealocytes. (A,B) Cells were treated with FSK and the indicated concentrations of K^+. (C,D) Cells were treated with FSK and the indicated concentration of PMA with K^+ (closed symbols) and without (open symbols) K^+ (15 mM).

mM) and FSK, PMA produced a concentration-dependent potentiation of the stimulation of cGMP (fig.3D). Two other PKC activators, 4β-phorbol 12,13-dibutyrate (PDBu) and dioctanoylglycerol (DC8) [33] also potentiated FSK + K^+ stimulation of cAMP and cGMP accumulation (table 1, expt II). In contrast, related compounds which do not activate pineal PKC, viz. 4α-phorbol 12,13-didecanoate (PDD) and diolien (DIOL) [33], did not cause potentiation (table 1, expt II). These findings indicate that PKC is involved in the PE → Ca^{2+} mechanism which potentiates FSK stimulation of cGMP accumulation.

Table 1

Effect of selected agents on K^+ potentiation of FSK-stimulated cAMP and cGMP accumulation in pinealocytes

Expt	Treatment	cAMP (pmol/mg protein)	cGMP (pmol/mg protein)
I	Control	21.1 ± 1.6	1.4 ± 0.8
	FSK	169 ± 4.2	14.3 ± 0.7
	+ K^+ (40 mM)	604 ± 37.3[a]	124 ± 12.7[a]
	+ K^+ (40 mM) + NIF	189 ± 13.1[b]	16.4 ± 2.1[b]
	+ NIF	174 ± 6.5	15.7 ± 2.0
	NIF	26.6 ± 3.2	1.7 ± 0.3
	K^+ (40 mM)	29.3 ± 2.4	2.1 ± 0.4
II	Control	18.3 ± 0.4	2.1 ± 0.3
	FSK	173 ± 16.9	15.6 ± 2.8
	FSK + K^+ (15 mM)	211 ± 15.4	21.2 ± 0.91
	+ PMA	2070 ± 85[b]	272 ± 16.4[b]
	+ PDBu	1530 ± 36[b]	250 ± 18.4[b]
	+ DC8	1000 ± 63[b]	116 ± 6.8[b]
	+ PDD	179 ± 21.3	23.0 ± 1.52
	+ DIOL	189 ± 15.6	17.0 ± 3.3

[a] Significantly different from the FSK-treated group ($p<0.05$)
[b] Significantly different from the corresponding FSK + K^+-treated group ($p<0.05$)

Expt I: NIF (1 μM) was added to some tubes and 5 min later FSK (100 μM) and K^+(40 mM) were added as indicated. 15 min later the cells were pelleted and frozen. Expt II: FSK (100 μM) K^+ (15 mM), PMA (0.1 μM), PDBu (0.1 μM), DC8 (100 μg/ml), PDD (0.1 μM) and DIOL (100 μg/ml) were added as indicated. 15 min later the cells were pelleted and frozen

4. DISCUSSION

The results of this series of experiments confirm previous reports that FSK can elevate cGMP [18,19], and demonstrate for the first time that effects of FSK on cGMP can be markedly enhanced by treatments which elevate $[Ca^{2+}]_i$ and translocate PKC. This leads to the suggestion that effects of FSK on cGMP accumulation in other tissues might be unmasked by cotreatment with appropriate agents; such a strategy might reveal the presence in these tissues of biochemical AND gates which physiologically regulate cGMP production.

Although there is clear evidence for a FSK →

cGMP link, the precise mechanism through which FSK acts to elevate cGMP is not clear from these studies. One explanation is that the cGMP response is secondary to that of cAMP. This seems unlikely because we have found that treatment with 0.1 mM dibutyryl cAMP does not elevate cGMP significantly (unpublished). Another possibility is that the cGMP response is independent of cAMP, and that guanylyl cyclase, like adenylyl cyclase, is activated by FSK. Other evidence of a similarity is that in the pinealocyte both enzymes appear to be regulated by G_s and PKC-dependent mechanisms in the pinealocyte [10,28], are substrates for PKC [12,23] and act on similar substrates to produce similar products. Accordingly, we suspect that pineal adenylyl and guanylyl cyclases belong to the same gene family.

Based on the findings here and elsewhere [18,19], it seems prudent to suspect that any effect of FSK treatment may in part reflect an action of cGMP. For example, very early reports on FSK describe blood pressure lowering effects [34–36]. Perhaps these effects reflect an action of cGMP, which is thought to be involved in the action of a well established and widely used blood pressure lowering agent, sodium nitroprusside.

Finally, it should be added that a number of convincing reports of cAMP-independent effects of FSK have appeared [37–39]. In view of these reports and the effects of FSK on cGMP, it is now apparent that the FSK = cAMP equation must be used cautiously.

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